Nucleic Acids Research, 1996, Vol. 24, No. 13 **2623–2625**

PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR

Susan J. Ahn, José Costa and Janet Rettig Emanuel*

Department of Pathology, Yale University School of Medicine, PO Box 208023, Lauder Hall 219C, New Haven, CT 06520-8023, USA

Received January 2, 1996; Revised and Accepted May 2, 1996

PCR has become a powerful tool for genetic analysis and many applications for gene sequence quantitation are based on this technology (1–3). Standardized reaction conditions require accurate quantitation of input DNA as well as optimization of chemical and cycling parameters. In this study we discuss PicoGreen™ (Molecular Probes, Eugene, OR) fluorescence enhancement as a useful assay for template DNA quantitation and PCR product formation.

Spectrophotometry is the principal method for evaluating quantity and quality of nucleic acids. In aqueous solution, DNA has maximal absorbance near 260 nm with an extinction coefficient of 50; protein absorbs light strongly near 280 nm. The concentration of a sample can be read directly (in μg/μl) by diluting it 1:20 in water or buffer; a practical lower limit of detection is 50–100 ng DNA in a 50–100 μl microcuvette. The A_{260}/A_{280} ratio provides an estimate of DNA purity; values of 1.7–2.0 predict 'clean DNA'. However, single-stranded DNA, RNA, PCR primers and dNTPs, or aromatic organic compounds such as phenol interfere by absorbing light in this range. Fixed tissue samples with substantial protein crosslinking and DNA preparations containing added enzymes or protein stabilizers are difficult to evaluate spectrophotometrically (4).

Intercalating fluorochromes, such as ethidium bromide or Hoechst 33258, selectively bind to dsDNA. The sensitivity of Hoechst 33258 is ~25 ng of DNA per assay, but preferential association with domains of high A–T content or reduced binding to DNA fragments <500 bp may result in skewed analysis (5). Accurate evaluation may require sophisticated or dedicated equipment since both dyes photobleach easily and fluorescence enhancement of DNA binding is low, leading to high background readings. These compounds are carcinogenic and pose handling and disposal problems.

Electrophoretic array is the most common means of evaluating molecular distribution of both simple and complex DNA samples. When stained with ethidium bromide, transillumination with 254 nm UV light permits CCD camera visualization of a single agarose gel band containing ~5 ng or a polydisperse sample containing 25–50 ng of dsDNA. SYBR-Green I™ (Molecular Probes, Eugene, OR) is a proprietary fluorescent dsDNA-specific stain that has an emission peak at 520 nm following excitation at 254 or 497 nm. Image collection and analysis with 254 nm transillumination requires the use of an optical quality band-pass filter to eliminate infrared interference. SYBR-Green I is more sensitive than ethidium bromide with a limit detection of ~50 pg per band or ~250 pg per lane polydisperse dsDNA. Argon laser-activated gel scanning or capillary electrophoresis is more sensitive (6), but far more costly. Gel analysis allows evaluation of genomic DNA integrity, completeness of restriction endonuclease digestion and quantity of late cycle PCR products. However, this method is impractical for routine or high throughput DNA quantitation (7).

PicoGreen is a fluorochrome that selectively binds dsDNA and has characteristics similar to that of SYBR-Green I. It has an excitation maximum at 480 nm (lesser peaks in the short-wave UV range) and an emission peak at 520 nm. When bound to dsDNA, fluorescence enhancement of PicoGreen is exceptionally high; little background occurs since the unbound dye has virtually no fluorescence. PicoGreen is very stable to photobleaching, allowing longer exposure times and assay flexibility. However, the molecular structure of the dye is proprietary and the mode of binding is not fully characterized, so potential handling hazard is unknown. We evaluated PicoGreen for quantitation of multiple DNA sample types. We examined the linearity of binding and the effective detection range for different species of 'high molecular weight' DNA standards (human placental, calf thymus and λ phage; with or without restriction digestion) and DNA isolated from a variety of tissue types preserved under different protocols. We also assayed 'low molecular weight' dsDNA (~150 bp PCR products) in the presence or absence of reaction primers, dNTPs and *Taq* polymerase. Oligonucleotide primers were evaluated for interference with quantitation in some samples.

Control DNAs were from commercial sources. *Eco*RI (New England Biolabs, Beverly, MA) digests were performed with 5 U per sample in a 10 μl reaction mix at 37°C for 2 h. We obtained sample DNA by organic extraction from flash-frozen or paraformaldehyde-fixed paraffin-embedded surgical remainder tissues. PCR mixtures contained 0.2 μM each primer, 50 μM each dNTP, 0.02 U/μl AmpliTaq polymerase (Perkin-Elmer Corp., Wilton, CT) and TaqStart MAb (Clontech, Palo Alto, CA). Primers were removed from PCR reactions with Microcon 30 (Amicon, Beverly, MA).

The A_{260}/A_{280} of each sample was read against a TE blank in a Lambda-2 Spectrophotometer (Perkin Elmer Corp., Norwalk, CT), fitted with a 100 μl quartz microcuvette. DNA samples were diluted 1 μl into 100 μl of TE. A reading of 0.020, the lower confidence level of the instrument, represented 100 ng of DNA

* To whom correspondence should be addressed

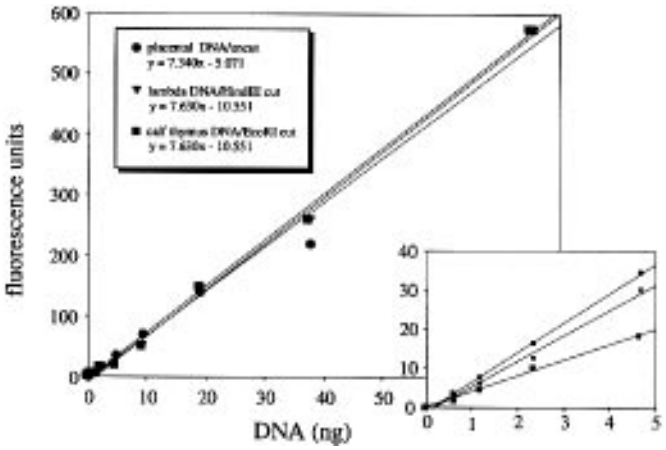

Figure 1. Linearity of PicoGreen assay. Commercial DNA preparations with differing sequence complexity and molecular size were serially diluted and assayed for linearity and sensitivity of detection. Assay conditions are as outlined in the text. The total amount of DNA, in ng per assay well, is plotted against fluorescence units recorded. The inset shows an expanded scale for DNA values <10 ng; the value of a 10 µl TE blank was subtracted as background.

in the cuvette and a concentration of 100 ng/µl DNA in the original sample.

Ethidium bromide was diluted to a final concentration of 0.2 µg/ml and SYBR-Green I was diluted 1/10 000 for staining of agarose gels. Electrophoresis was at 150 V for 1 h in an MPH apparatus (IBI, New Haven, CT). An IS-1000 Digital Imager (Alpha-Innotec, San Leandro, CA) with an EB-1 or SG-3 band pass filter collected images generated by 254 nm UV transillumination. Quantitation standards were commercially purchased genomic DNA or a 100 bp ladder marker (Research Genetics, Huntington, AL) calibrated for DNA content (10 ng/µl per band) as well as fragment size.

PicoGreen dye was diluted 1:200 with TE, pH 7.4; each reaction contained 140 µl of dye solution plus a sample DNA made up to 10 µl in TE. Standard curves were constructed by serial dilution of DNA, based on quantitation by the commercial provider. Microtiter plates were read in LS50B Spectrofluorimeter with plate reading attachment (Perkin-Elmer/Applied Biosystems, Foster City, CA) or CytoFluor II Plate Reader (Perseptive Biosystems, Framingham, MA). PicoGreen-stained samples were excited at 485 nm; emission at 520 or 530 mn was recorded. Triplicate aliquots were evaluated for each point between 0.060 and 600 ng and averages were graphed. Background and blank values were subtracted and data plotted and compared. Assay sensitivity was also evaluated by spot densitometry; aliquots of 25 µl reaction solution were spotted on Saran laid over the UV-transillumination source with data collection by CCD camera. Sensitivity limits and assay linearity were comparable with microtiter format, but sample handling and data capture were cumbersome (data not shown). Figure 1 shows that PicoGreen detection of DNA is linear at this dye concentration over the range of 0.25–150 ng. Neither fragment size nor complexity of DNA influenced the assay since eukaryotic genomic DNA (restricted or intact) λ phage DNA and 150 bp PCR product bind PicoGreen with the same sensitivity and efficiency. This figure also illustrates that the presence of protein (restriction endonuclease) in the sample only affects the PicoGreen assay in the 0.25–5.0 ng DNA range.

The quantitation of either high or low molecular weight genomic DNA by PicoGreen more closely represents the evaluation of DNA visualized in gel array than measured by spectrophotometric analysis. Figure 2 demonstrates that standard A_{260}/A_{280} quantitation may deviate widely from visualized dsDNA in each sample as arrayed in an ethidium bromide-stained gel. PicoGreen analysis better represents the sample, even when a PCR product as small as 150 bp is assayed. Although we isolate all DNA by the same method, variations in tissue processing often produce inconsistencies in spectrophotometric evaluation. Miscalculation of genomic DNA quantity can result in anomalous results in subsequent molecular biological manipulations such as restriction digest analysis, Southern blotting and PCR. Figure 2 also compares the utility of spectrophotometry and PicoGreen assay for samples containing restriction enzyme or ssDNA PCR primers. Whereas PicoGreen allows efficient quantitation of low concentration DNA in the presence of moderate levels of restriction enzyme, spectrophotometric analysis is impossible; the presence of standard concentration (0.2 µM) oligonucleotide primers minimally influenced PCR product quantitation.

Table 1A demonstrates that PCR product quantitation of <100 ng DNA by spectrophotometry is ruled out by the inability to discriminate dsDNA in the presence of primers and polymerase; whereas PicoGreen assay shows interference only at product levels <1.0 ng (~10 fmol). Table 1B shows that the contribution of primers to fluor detection assay is negligible at the concentrations (<0.2 µM) typically used for PCR.

Table 1. PCR master mix components and DNA product quantitation

A. Effect of master mix on PCR product quantitation

	PCR–		PCR+	
	Pico	Spec	Pico	Spec
A	7.17	5*	8.50	120
B	4.12	0	6.71	125
C	2.42	0	3.51	110
D	1.66	0	2.71	115
E	1.24	0	2.21	130

B. Effect of primer concentration on PCR product fluorescence

Primer conc (µM)	Primers alone	Primers + PCR product
5.0	11.26	87.42
2.0	4.06	86.02
1.0	2.18	84.52
0.5	0.00	83.92

(**A**) One PCR reaction was divided, and primers and dNTPs were removed from half by Microcon-30; each portion was serially diluted and assayed. PCR– samples had primers removed and were diluted with TE; PCR+ samples contained the original reaction mix and were diluted with fresh PCR master mix to maintain primer and dNTP concentrations. (**B**) Primers were included in a PCR master mix at various final concentrations and assayed by PicoGreen in the absence or presence of 7.5 ng (~1 µM) purified PCR product. Pico values are fluorescence units above background; Spec values represent ng DNA.

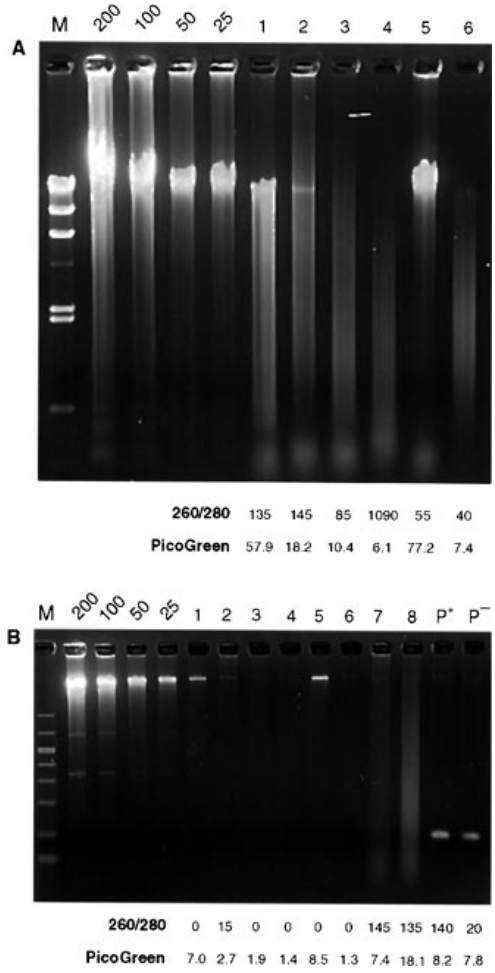

Figure 2. Comparison of DNA quantitation assays. **(A)** High molecular weight DNAs; **(B)** low molecular weight DNAs. Genomic DNA from snap-frozen tissue was assayed directly (A1–A6) or after *Eco*RI digestion (B1–B6); DNA from fixed and embedded tissue was assayed directly (B7 and B8). A 151 bp PCR product was analyzed directly (P+) or after removal of PCR primers and dNTPs (P–). Replicate 1 µl aliquots of each sample were assayed by each technique. Ethidium bromide gel conditions: (A) 1% agarose gel, markers = λ DNA cut with *Hin*dIII; (**B**) 2% agarose gel, markers = a 100 bp ladder. Commercial DNAs were diluted as reference standards; values are ng DNA per lane. Values below gel lanes represent ng DNA as assayed by A_{260} and PicoGreen.

In Table 2 we compare the various discriminators and formats that we have reviewed or demonstrated. We conclude that PicoGreen staining for quantitation of dsDNA is a simple, sensitive and practical alternative to evaluation by spectrophotometric absorbance at 260 nm, fluorogenic intercalation of ethidium bromide and Hoechst 33258 dye, or staining of electrophoretically arrayed samples. PicoGreen assays have a threshold of <1.0 ng dsDNA per sample, regardless of the detection equipment, or molecular complexity of the sample; ~250 ng per sample of genomic DNA and ~1.0 ng small PCR product are the practical limits of the assay in our microtiter format. Fluorescence enhancement of the dye upon binding dsDNA is substantial, producing very little background. Compounds that skew conventional assays (protein, ssDNA, RNA, phenol) have minimal effect; direct assay is possible after restriction digestion or in amplification mix. It is the most reliable method we have found for quantitation of PCR template DNA from archival or obscure patient samples. PicoGreen assay may provide direct quantitation of PCR product or analysis before sequencing or SSCP, if the reaction employs low primer and dNTP concentrations and produces a clean product without primer artifacts.

Table 2. Practical limits of DNA quantitation

Assay	Format	Amount of DNA (ng)	Assay volume (µl)	Notes
Ethidium bromide	gel	25–50	20	polydisperse
		5	20	single band
SYBR-Green I	gel	0.25	20	polydisperse
		0.05	20	single band
A_{260}/A_{280}	solution	100	100	microcuvette
Hoechst	solution	50	200	microwell
PicoGreen	solution	0.25–0.5	150	microwell

The various detectors and formats of DNA quantitation are compared for practical limits of detection using the methods discussed in this paper.

REFERENCES

1 Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Ehrlich, H.A. (1988) *Science* **239**, 487–491.
2 Ferre, F. (1992) *PCR Methods Appl.* **2**, 1–9.
3 Clementi, M., Menzo, S., Bagnarelli, P., Manzin, A., Valenza, A. and Varaldo, P.E. (1993) *PCR Methods Appl.* **2**, 191–196.
4 Sambrook, J., Fritsch, E.F. and Maniatis, T. (eds) (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY.
5 Cesarone, C.F., Bolognesi, C. and Santi, L. (1979) *Anal. Biochem.* **100**, 188–197.
6 Skeidsvoll, J. and Ueland, P.M. (1995) *Anal. Biochem.* **231**, 359–365.
7 Sharp, P.A., Sudgen, B. and Sambrook, J. (1973) *Biochemistry* **12**, 3055–3063.